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AN ELECTRON MICROSCOPIC STUDY OF HEMOPOIETIC  
TISSUES IN THE COURSE OF LEWIS LUNG CARCINOMA

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Abstract

Lewis lung carcinoma of C57Bl/6 mice causes a progressive anemia with reticulocytosis and splenomegaly, metastasizes to lungs, liver, and kidneys but does not invade hemopoietic tissues. The cause of this anemia is uncertain. We studied the structure of spleen and bone marrow in these tumor hosts by light and electron microscopy. Splenic congestion of the red pulp with numerous erythropoietic islands and marrow hyperplasia characterized the hemopoietic tissues of these mice, which, when coupled with other hematological parameters suggested a hemolytic condition. However, the erythropoietic response appeared to be in part ineffective as evidenced by phagocytosis of immature as well as mature red blood cells within the spleen and marrow. Thus, the condition of anemia in Lewis lung carcinoma may result from a multifactorial response of hemolysis and ineffective erythropoiesis and hemolysis.

Introduction

Lewis lung carcinoma (LLC) is an experimental tumor which can be maintained by subcutaneous transfer of tumor cells in C57Bl/6 mice (7, 9). It metastasizes to the lungs, liver and kidneys but not to hemopoietic organs (7). However, during the course of the disease the spleen becomes progressively and massively enlarged and the animals develop progressive anemia with reticulocytosis, suggesting hemolysis (1, 4, 5). To gain further insight into the mechanism of this anemia, the structure of spleen and bone marrow of these tumor-bearing animals was studied by light and electron microscopy. The results support the suggestion of hemolysis and further indicated ineffective erythropoiesis.

Materials and Methods

Tumor System

LLC was obtained from the Frederick Cancer Research Facility (Frederick, MD) and has been maintained in our laboratory by subcutaneous implantation of 2 mm tumor fragments every 14 days into male C57Bl/6 mice, 18-20 grams (NCI). The method for its passage and maintenance and quality control has been described in detail (1, 4) and will not be repeated here. In the present work, each mouse received a single-cell suspension of  $10^6$  viable LLC cells resuspended in Hanks' Balanced Salt Solution (HBSS). Our previous work has shown that the implantation of this cell number results in 100% "take" with a median survival of 23 days. Control animals received an injection of equal volume of HBSS. This group is referred to here as "sham controls."

Hematologic Studies

At various times after tumor implantation, groups of animals (at least 5 per group) were sacrificed for hematologic studies. Heparinized whole blood was collected by cardiac puncture; hemoglobin, hematocrit, reticulocyte, total WBC, and differential WBC counts were determined. Reticulocyte counts were corrected for the hematocrit. Femoral bone marrow was harvested and total cell number per femur was determined and was not different in tumor-bearing and control animals (1, 4); a myeloid: erythroid ratio was ascertained on smears by counting 500 cells. Splenic weight of each mouse was recorded at the time of marrow harvest.

Microscopic Studies

For light microscopy spleen and marrow tissues from at least 5 animals per group were fixed in 10%

Key Words: Anemia, Lewis lung carcinoma, murine tumor system, cancer, spleen, electron microscopy, tumor-induced, hemopoiesis, erythropoiesis, erythroclasia, hemolysis.

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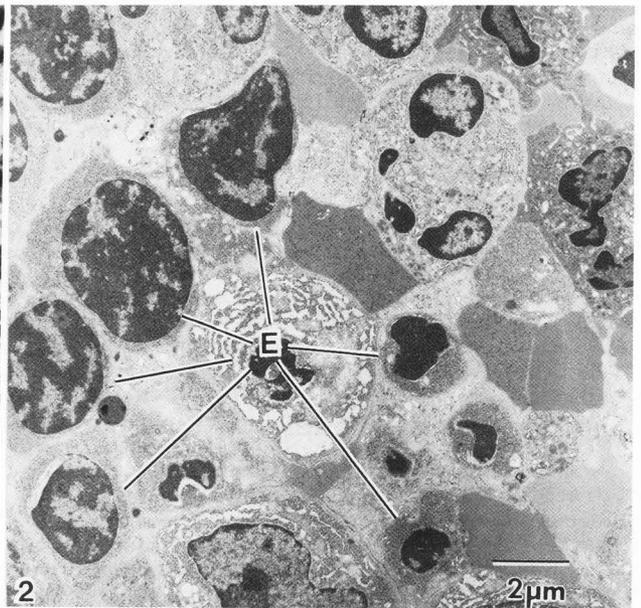
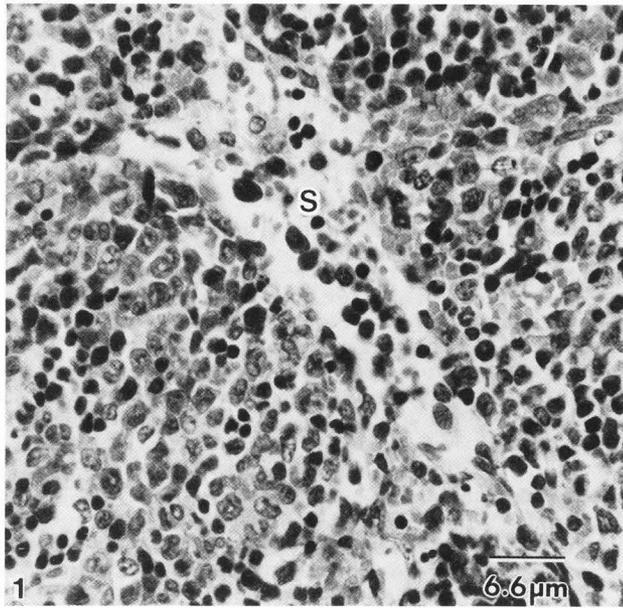


Figure 1. Light micrograph of a hematoxylin and eosin stained section of the spleen of an LLC-bearing mouse 19 days post tumor implantation. Note congestion of the red pulp with numerous foci of hemopoiesis. Splenic sinuses contained evidence of intravascular erythropoiesis. (S) sinus.

Figure 2. Electron micrograph of a section of red pulp of a spleen from an LLC mouse 14 days post implantation. Erythroblastic islands such as this one on which a central macrophage forms a focus of erythropoietic activity are seen throughout the red pulp. (E) erythroid cell.

Table 1. Hematological parameters in LLC mice and sham controls 19 days post tumor implantation.

	LLC	Sham Controls
Neutrophils/ $\mu$ l ( $\times 10^{-3}$ )	32,110 $\pm$ 8200	2,514 $\pm$ 1100
Hemoglobin (g/dl)	5.5 $\pm$ 0.3	12.0 $\pm$ 0.8
Hematocrit	18 $\pm$ 1.9	37 $\pm$ 2.7
Reticulocytes (%)	8.3 $\pm$ 3.1	1.7 $\pm$ 0.7
Marrow Myeloid: Erythroid Ratio	1.5:1	1.2:1
Spleen Weight (mg)	362 $\pm$ 27.9	77 $\pm$ 1.5

formalin, buffered with phosphate buffered saline (pH 7.4), dehydrated in ethanol, and embedded with paraffin. Sections (3 microns in thickness) were stained with hematoxylin and eosin.

For transmission electron microscopy (TEM) spleens and marrow tissues from at least 5 animals per group were removed, cut into small pieces about 1-2 mm which gave approximately 4 samples for processing per tissue and fixed in 4% cold glutaraldehyde in cacodylate buffer (pH 7.3) overnight at 4°C. The tissues were then post-fixed in similarly buffered 1% cold osmium tetroxide for 45 minutes at 4°C. They were dehydrated in a graded series of

ethanol, infiltrated with Epon 812, and embedded in Epon 812. The samples were cured for 3 days in 40°C and 60°C ovens and then were sectioned. Sections of 1 micrometer thickness were obtained with glass knives stained with alkaline toluidine blue (pH 11) and examined by light microscopy. Suitable areas were then trimmed and thin sectioned with a diamond knife in an LKB-5 microtome. Sections were stained with 2.5% aqueous uranyl acetate for 1 h and 0.2% lead citrate for 5 minutes and observed in a JEOL 100 CX electron microscope (60 kV).

### Results

#### Hematological parameters

Hematological parameters in tumor-bearing mice and sham controls are shown in Table 1. LLC mice developed significant anemia represented by a decline in hemoglobin concentration (LLC 5.5  $\pm$  0.3 vs. control 12.0  $\pm$  0.8) and a hematocrit (LLC 18  $\pm$  1.9 vs. control 37  $\pm$  2.7).

This anemia became apparent by day 3 (hemoglobin 10.1  $\pm$  0.3 in LLC) and progressively worsened with growth of the tumor. Significant reticulocytosis also developed with counts becoming as high as 8.3  $\pm$  3.1 in tumor-bearing mice. In addition, leukocytosis with neutrophilia progressed from day 3 until the death of the animal (LLC 32,110  $\pm$  8200, control 2,514  $\pm$  1100). In the bone marrow the myeloid:erythroid (M:E) ratio went from 1.2:1 in controls to as high as 3.9:1 in LLC mice by day 7. By day 19 the M:E ratio in tumor-bearing animals approached that of control. Significant splenomegaly developed progressively in LLC mice with the spleen increasing in size about 5 times (362  $\pm$  27.9 vs. 77  $\pm$  1.5 mg).

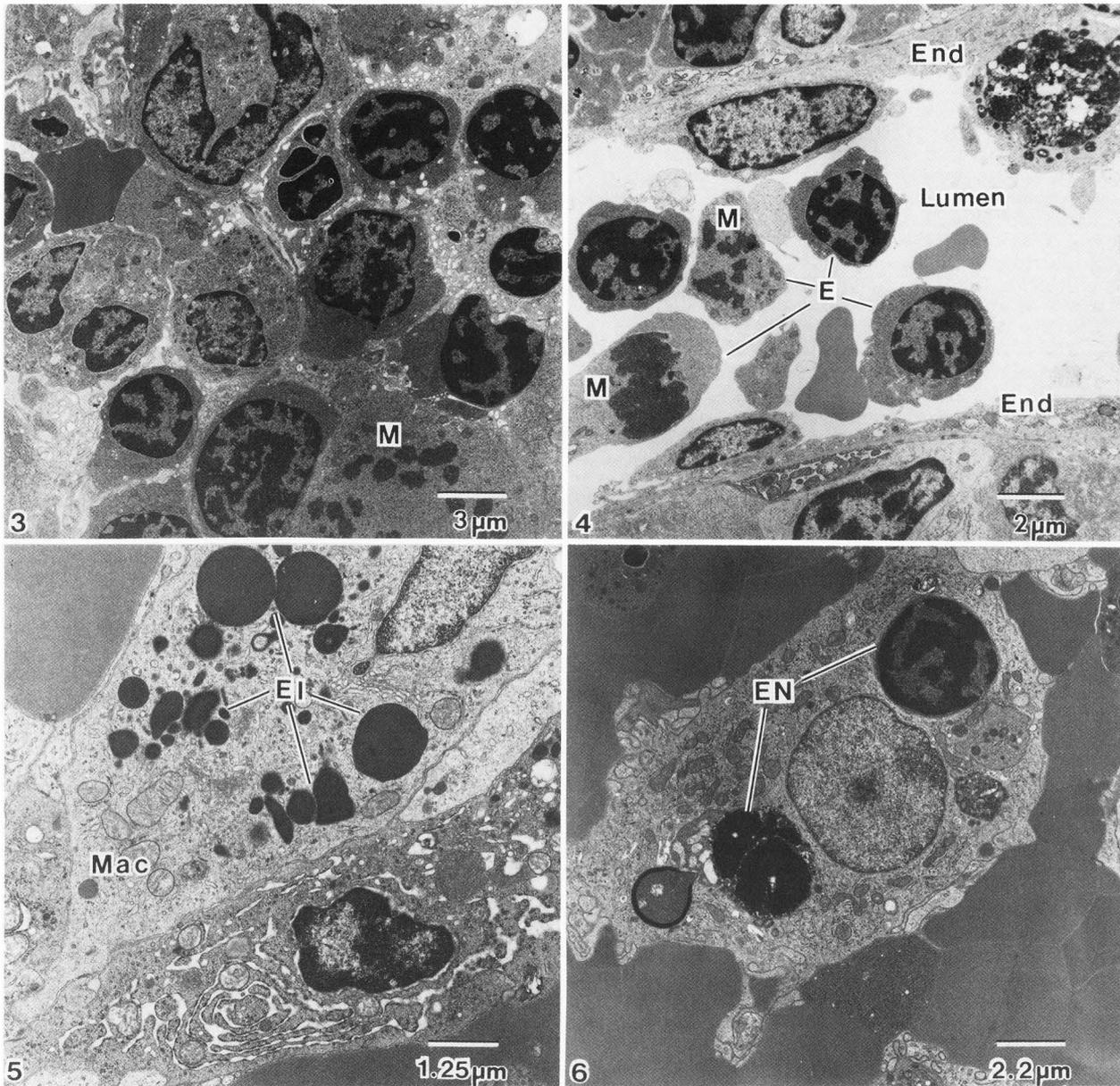


Figure 3. Electron micrograph of a section of bone marrow from the same mouse as the spleen in figure 2. Erythroblastic islands such as these were present in the marrow, with mitotic figures frequently seen. (M) mitosis.

Figure 4. Electron micrograph of a splenic sinus from the spleen of a 14 day tumor-bearing mouse. Note evidence of active intraluminal erythropoiesis, with developing erythroid cells and mitoses. (M) mitosis, (E) erythroid cell, (End) endothelial cell.

Figure 5. Electron micrograph of a section of splenic red pulp of an LLC mouse 11 days post implantation. Macrophages such as this one were frequently seen in the process of digesting hemoglobinized red cell fragments. (Mac) macrophage, (EI) erythroid inclusion.

Figure 6. Electron micrograph of another splenic section from an LLC mouse, 7 days post implantation. A nucleus as well as hemoglobinized cell fragments of an erythroid cell have been ingested by a cordal macrophage. (EN) erythroid nucleus.

#### Microscopic findings

By light microscopy the splenic red pulp, as well as marrow of tumor-bearing mice appeared markedly congested and hypercellular, with splenic cords containing numerous foci of hemopoiesis (Figure 1). This included all three lineages (erythropoiesis, granulopoiesis, and megakaryocytopoiesis). By comparison, the white pulp appeared somewhat small and hypotrophic. This congestion of red pulp progressed with growth of the tumor. Splenic sinuses contained many mature red cells but also numerous hemopoietic foci, indicating the occurrence of intravascular erythropoiesis within the sinuses of spleen in tumor-bearing mice (Figure 1). Foci of hemopoiesis were also seen in electron microscopic preparations of both spleen and marrow (Figures 2, 3). Erythropoietic foci usually could be perceived as surrounding a central macrophage, thus forming an erythroblastic island (2). Branches of central macrophages penetrated between developing erythroid cells which were often in the synchronous maturational stage. The central macrophage often contained red cell nuclei and sometimes entire nucleated erythroid cells. Mitotic figures were frequently seen in these islands (Figure 3).

Intravascular erythropoiesis was also observed within the lumen of splenic sinuses, and again developing erythroid cells were in synchronous maturational stages and mitoses not infrequently seen (Figure 4).

Of much interest was the magnitude of phagocytosis by cordal macrophages (Figures 5, 6). This included both hemoglobinized fragments of erythroid cell cytoplasm as well as their nuclei and the entire cell. This erythrophagocytosis was the salient feature of spleen particularly after day 7 post implantation. It was striking when compared to the spleen from sham control mice. No tumor cells were seen in marrow or spleen at anytime during the course of tumor development.

#### Discussion

The profound anemia that develops during the course of this experimental tumor is associated with progressive reticulocytosis, marrow hyperplasia and splenomegaly and suggest a hemolytic disorder. Neither the spleen nor the marrow is infiltrated by tumor. This is evident not only by the absence of tumor cells in these organs in our present microscopic examination, but also by functional studies in which organ cell suspensions are subcutaneously implanted in new hosts. These implants never lead to tumor production. Hence, splenomegaly and marrow hyperplasia, observed in this study, should be interpreted in the context of anemia and other hematologic abnormalities as outlined in Table 1.

In other experimental tumors, Wieman et al. (10) have shown the production of a hemolytic substance which can lyse normal red cells in vitro. Similarly, in clinical situations, hemolytic anemias are frequently associated with the presence of solid tumors (3, 6, 8) as well as hematological malignancies. Further studies on the mechanism of hemolysis in this experimental tumor may shed some light on the mechanism of tumor-associated hemolytic disease in humans.

Of further interest in this study was that erythropoietic response to hemolysis appeared to be

at least in part ineffective. This is evident by phagocytosis of immature cells in spleen as well as bone marrow. The reason for this ineffective erythropoiesis is not clear. It is possible, however, that the hemolytic factor responsible for destruction of mature cells may extend its effect to immature ones, thus leading to ineffective erythropoiesis.

#### Acknowledgements

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(see facing page for discussion with reviewers)

## Anemia in Lewis Lung Carcinoma

### Discussion with Reviewers

H. Gamliel: If Lewis lung carcinoma invades the liver and kidneys too, why was "lung" (and not liver, etc.) attributed to this type of carcinoma? Can you speculate on why LLC invades the lungs, liver, and kidneys but not hemopoietic tissues? Studies of such a kind might contribute a lot in shedding more light on the mechanism of tumor infiltration. Are you aware of such studies?

Authors: This is a very interesting question. LLC can potentially provide an excellent model to study the mechanism of metastasis in solid tumors. This is because of its highly predictable pattern of metastasis. However, to our knowledge, this model system has not been adequately exploited. Lung, (rather than liver and kidney) metastasis is generally emphasized because it is the earliest and most predictable one. We feel this occurs because of the invasion of the venous system for which the lung is the first stop in the course of circulation.

H. Gamliel: It seems that the description of erythropoietic foci surrounding a central macrophage is referring to the spleen only. If this is correct, are there comparable patterns of formation of such foci in the bone marrow?

Authors: Yes, both tissues are involved.

H. Gamliel: There is only limited information on the microstructure of the bone marrow in the studied samples. Compared to the describe changes in red and white pulp of the spleen, are there any such changes in the stroma of the bone marrow?

Authors: We have not observed such changes and consequently we have not described any.

H. Gamliel: Did you notice any macrophages or macrophage "branches" in close relation to granulopoiesis or megakaryocytopoiesis foci? Did you ever see fragments of myeloid or mega cells in the macrophages' cytoplasm? If not, what were your criteria for differentiating between immature erythroid fragments and pieces from other immature blood cells? Compared to the "ineffective erythropoiesis," would you say that the mega- and/or granulopoiesis were (super) effective, as might be indicated in Table 1?

Authors: Probably ineffective hemopoiesis involves all three cell lines (erythropoiesis as well as granulopoiesis and megakaryocytopoiesis). However, the magnitude of granulopoiesis is so intense that the net result is blood granulocytosis. This intense granulopoiesis can be attributed to growth factor production by tumor cells as we have reported before (1, 4).

The mechanism of thrombocytopenia has always intrigued us. How much ineffective thrombocytopoiesis contributes to this is not known. This is an area that is currently under study.

T. Seed: Is the early rise (at 7 days) in the M/E ratio due to enhanced granulopoiesis or suppressed erythropoiesis, or both?

Authors: This is due to enhanced granulopoiesis. We have previously reported (1,4) on this aspect. Enhanced granulopoiesis in the course of this tumor is biphasic, with early and late phases. Both are the result of the production of GM-CSF and M-CSF by the tumor.

T. Seed: Can LLC cells (or their soluble products) directly mediate, in vitro, similar proliferative/suppressive responses?

Authors: Yes. We have developed a cloned cell line from this tumor under serum-free conditions. Our data, as yet unpublished, indicate the elaboration of granulopoietic factors by this cell line. Whether there is a suppressive factor as well is currently under study.

M. Kashimura: Congestion is usually used when venous blood stagnates. I doubt whether congestion is a proper word when the red pulp is occupied with erythroblastic islands.

Authors: This definition could be applicable to those organs where the circulation is closed. In spleen with its characteristically open circulation, the situation is entirely different. Here the term has been used in any condition that leads to blockage of cordal pathways.